

## Distribution of orbicules in Annonaceae mirrors evolutionary trend in angiosperms

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**Background and aims** – Orbicules or Ubisch bodies have been recorded in many angiosperm families and although the first observations date back to 1865, their function in the anther remains enigmatic. In flowering plants a general evolutionary trend is observed from common occurrence of orbicules in early diverging lineages towards a more patchy distribution in derived clades of eudicots. Annonaceae was our family of choice for an in depth study of orbicule distribution in early diverging angiosperms since it met the following three criteria: (1) high tapetum diversity, (2) orbicule presence and absence recorded, and (3) recent phylogeny at genus level available.

**Key results** – Our SEM data of eighteen species show that orbicules are more common in Annonaceae than previously perceived. The resulting orbicule distribution pattern on the family topology indicates a consistent absence of orbicules in the ‘long branch clade’ while orbicules are present in *Anaxagorea*, the ambavioids, and the ‘short branch clade’. Presence of orbicules is the ancestral condition in the family. Morphologically, Annonaceae orbicules are small (< 1 µm), spherical and smooth.

**Conclusions** – The orbicule distribution pattern in Annonaceae reflects the general evolutionary trend in flowering plants. We hypothesize that orbicule presence can be considered as a powerful proxy for non-amoeboid tapetum characterization in Annonaceae.

**Key words** – Annonaceae, orbicules, palynology, sporopollenin, starch granules, tapetum, Ubisch bodies.

### INTRODUCTION

Successful sexual reproduction in flowering plants requires the development of viable microspores in the anther locules. Pollen grains assure safe transport of the male gametes to the female reproductive organs by their elaborate outer wall of sporopollenin (Scott 1994). The source of sporopollenin precursors and the mechanism by which they polymerize in a genetically determined pattern, remain challenging research topics in palynology (Blackmore et al. 2007). Next to the pollen grain exines, polymerized sporopollenin can also be found in the form of orbicules on the inner locule wall. Orbicules or Ubisch bodies are a-cellular sporopollenin particles, usually < 1 µm to few micrometres in diameter that line the tapetal membrane. They originate as lipid droplets in the tapetal cytoplasm associated with the rough endoplasmic reticulum (pro-orbicules; e.g. El-Ghazaly & Jensen 1986) and acquire their sporopollenin wall after exocytosis into the locular space synchronously with microspore wall development

(Christensen et al. 1972, El-Ghazaly & Huysmans 2001). The function of orbicules is unknown today (see Huysmans et al. 1998 for a review). Already in the very first papers on orbicules (Rosanoff 1865, von Ubisch 1927, von Kosmath 1927) a positive correlation was hypothesized between orbicule production and a parietal (syn.: secretory) tapetum type. Recently the actual distribution of orbicules in angiosperms was reviewed (Huysmans et al. *subm.*). It is demonstrated that orbicules are a plesiomorphic character in flowering plants with an almost common occurrence in the ANITA grade and the early diverging lineages of all major groups in angiosperms. Orchidaceae and Asteraceae typically lack orbicules. Orbicule presence/absence is remarkably constant at family level: in only twenty angiosperm families both positive and negative observations were recorded (Huysmans et al. *subm.*).

As a complement to our previous orbicule studies in Rubiaceae (Gentianales, euasterids I; Huysmans et al. 1997, Vinckier et al. 2000, Dessein et al. 2005, Verstraete et al.

subm.), Annonaceae was our family of choice in the magnoliids. Annonaceae met the following three criteria : (1) both presence and absence of orbicules were recorded, (2) a high level of tapetum diversity is present, and (3) a recent phylogeny based on molecular data at generic level is available. Annonaceae are the largest family of the Magnoliales (Sauquet et al. 2003) and comprise 2500 species of trees and lianas, mainly found in lowland tropical forests of all continents (Chatrou et al. 2004). A phylogenetic framework has been put forward in a number of papers in recent years (e.g. Mols et al. 2004, Richardson et al. 2004, Pirie et al. 2006, Couvreur et al. 2008, Chatrou et al. subm.). Despite considerable efforts to bring the classification of Annonaceae in line with current phylogenetic hypotheses (e.g. Mols et al. 2008, Zhou et al. 2009), there are still several non-monophyletic genera. The taxonomic level of these classificatory uncertainties is too shallow, however, to influence the results presented here.

Orbicules were believed to be absent in Annonaceae (as in Degeneriaceae) but considered present in Magnoliaceae by Bhandari (1971: 286). However, in a survey at angiosperm level (Huysmans et al. 1998 updated in Huysmans et al. 2000) two Annonaceae species with, and 21 species without orbicules were listed based on unpublished data (C.-H. Tsou, Institute of Botany, Taipei, Republic of China, unpubl. res.). Gabarayeva (1995) reported orbicules in *Anaxagorea brevipes* and their absence in *Asimina triloba* (Gabarayeva 1992, 1993). As a partial explanation of the presence/absence distribution of orbicules in Annonaceae, tapetum characterization is known to be crucial (see Huysmans et al. 1998 and Huysmans et al. subm. for a review). In basal angiosperms the dominant type of tapetum is undoubtedly secretory or parietal (Furness & Rudall 2001). We prefer the latter term since all tapetum cell types obviously retain a secretory function. Therefore the morphological terminology (parietal vs. amoeboid) seems more accurate here than the functional terminology (secretory vs. plasmodial). In the amoeboid type the tapetal cells loose their walls to form a plasmodium during microspore development that intrudes the locule to guarantee a direct physical contact between tapetum and microspores. In a parietal tapetum the cells keep their individuality and their position, thereby delimiting a locular space throughout maturation. Most likely the locular fluid acts as a transport medium for precursors, enzymes and other metabolites produced by the tapetum (Pacini et al. 1985, Pacini 2010). The invasive tapetum is an intermediate type with cells that keep their individuality but move into the locule during certain time intervals. This process might happen in a cyclic pattern as in *Nymphaea* for instance (Rowley et al. 1992).

The present study is designed to provide new relevant data of orbicule presence/absence in Annonaceae in order to increase our understanding of evolutionary patterns concerning orbicule distribution and morphology in flowering plants. We address the following three aims: (1) screen all major clades in Annonaceae for presence/absence of orbicules and document morphological variation; (2) optimize data on a recent phylogeny of the family to assess the phylogenetic signal in distribution pattern, and (3) explore correlation with tapetum type and discuss the data available for Magnoliales.

## MATERIALS AND METHODS

The present study is based on herbarium specimens and fixed material of the collections in U and WAG. Additional living material from the National Botanic Garden of Belgium was fixed and embedded for LM observations (see Appendix for material investigated). Sampling is carefully considered to represent all clades in the phylogeny of Annonaceae (sensu Chatrou et al. subm.). Of large (e.g. *Annona*) or polyphyletic genera (e.g. *Polyalthia*) several species were included in an attempt to cover the full range of variation.

### SEM-observation

Flowers or anthers were isolated from herbarium specimens and rehydrated for 12 h in Agepon (1:200 in distilled water). All other flower organs were discarded and anthers were dehydrated by a graded ethanol series (33%–50%–70%–95%–100%), prior to critical point drying (Leica EM CPD030, Bal-TEC AG, Balzers, Liechtenstein). For ethanol material the rehydration step was skipped and dehydration was continued from the stock solution. Dried anthers were fixed to aluminium stubs with double adhesive carbon tabs. If necessary excess pollen grains were gently removed using a cactus spine to clear part of the locule wall. Stubs were sputter coated with gold (Sputter coater, SPI supplies, West Chester, Pennsylvania, USA). Orbicules were observed using a SEM (JEOL JSM 6360, Jeol Ltd, Tokyo, Japan) at an accelerating voltage of 15 kV and a working distance of 10 mm. All measurements were made on digital REM images using Carnoy (Schols et al. 2002).

### LM-observation

Anthers were fixed in 2.5% glutaraldehyde in sodium cacodylate buffer (0.1M) during 8 h. Air bubbles were removed by vacuum extraction. After dehydration in a graded ethanol series, anthers were embedded in LR-white resin and polymerized at 60°C during 48 h. Sections were cut at c. 1 µm using an ultramicrotome (LKB2128 Ultratome, Bromma, Sweden) and stained with 0.1% toluidin blue. Observations were made with a Leica DMLB light microscope (Leica Microsystems Ltd., Heerbrugg, Switzerland).

### Character optimisation

Character states for orbicule presence/absence are extracted from literature and combined with the original observations in the present study (see table 2 for summary). A comparative study of orbicule distribution in flowering plants (Huysmans et al. subm.) demonstrated that at generic level the variation for the character presence/absence of orbicules is extremely low. Therefore extrapolation of data to generic level seems justified.

Presence/absence of orbicules was optimized under the equal and unordered weights criterion (Fitch parsimony; Fitch 1971), onto a randomly chosen tree topology from Chatrou et al. (subm.). The topology shows four major clades (fig. 4): (1) the genus *Anaxagorea* as sister to the remainder of the family; (2) the small clade ambavioids; (3) the ‘short branch clade’ comprising approx. 800 species and (4) the ‘long branch

**Table 1 – Summary of orbicule observations for all species studied.**

+ = present, – = absent; Density: a = abundant, s = scattered, va = very abundant; Diameter: minimum-(mean)-maximum; Ornamentation: ps = psilate, mg = microgranulate, perf = central perforation; threads = orbicules interconnected by threads, emb = embedded in tapetal remnants, agg = aggregated.

Species	Orbicules	Density	Diameter (µm)	Ornamentation	Notes	Figure
<i>Anaxagorea dolichocarpa</i>	+	s	0.42–(0.59)–0.77	ps	threads, emb	1A
<i>Annickia letestui</i>	+	va	0.33–(0.40)–0.47	ps	threads, emb	
<i>Annona cherimola</i>	–	–	–	–	–	2A–B
<i>A. muricata</i>	–	–	–	–	–	2C
<i>A. senegalensis</i> subsp. <i>oulotricha</i>	–	–	–	–	–	
<i>Asimina</i> × <i>nashii</i>	–	–	–	–	–	
<i>Cananga odorata</i>	+	va	0.44–(0.51)–0.62	ps, perf	agg	1E
<i>Crematosperma microcarpum</i>	+	va	0.33–(0.50)–0.65	ps, perf	–	1C–D
<i>Fissistigma latifolium</i>	–	–	–	–	–	2D
<i>Klarobelia cauliflora</i>	+	a	2.17–(2.62)–2.91	mg, perf	emb	1B
<i>Maasia sumatrana</i>	+	s	0.16–(0.29)–0.46	ps	–	1G
<i>Miliusa velutina</i>	+	a	0.46–(0.55)–0.64	ps, perf	emb	
<i>Monodora angolensis</i>	–	–	–	–	–	3A–C
<i>M. crispata</i>	+	va	0.23–(0.29)–0.39	ps	–	1F
<i>M. minor</i>	–	–	–	–	–	3D–E
<i>Mosannonna depressa</i>	+	s	0.67–(0.92)–1.13	ps	–	
<i>Oxandra xylopioides</i>	+	va	0.34–(0.50)–0.64	ps	threads	
<i>Polyalthia laterifolia</i>	+	va	0.57–(0.75)–0.93	mg	–	
<i>P. subcordata</i>	+	va	0.35–(0.43)–0.55	ps	–	1H

clade' with c. 1500 species. For this study a supermatrix was constructed using eight chloroplast DNA regions, and 200 species representing 96 out of 111 currently recognized genera. Before optimizing presence/absence of orbicules we simplified the tree to represent relationships amongst 89 genera. Character optimization was done using Mesquite 2.72 (Maddison & Maddison 2009). The polyphyletic genera *Polyalthia* and *Friesodielsia* are represented more than once in the tree.

## RESULTS

### Presence/absence and morphological diversification of orbicules

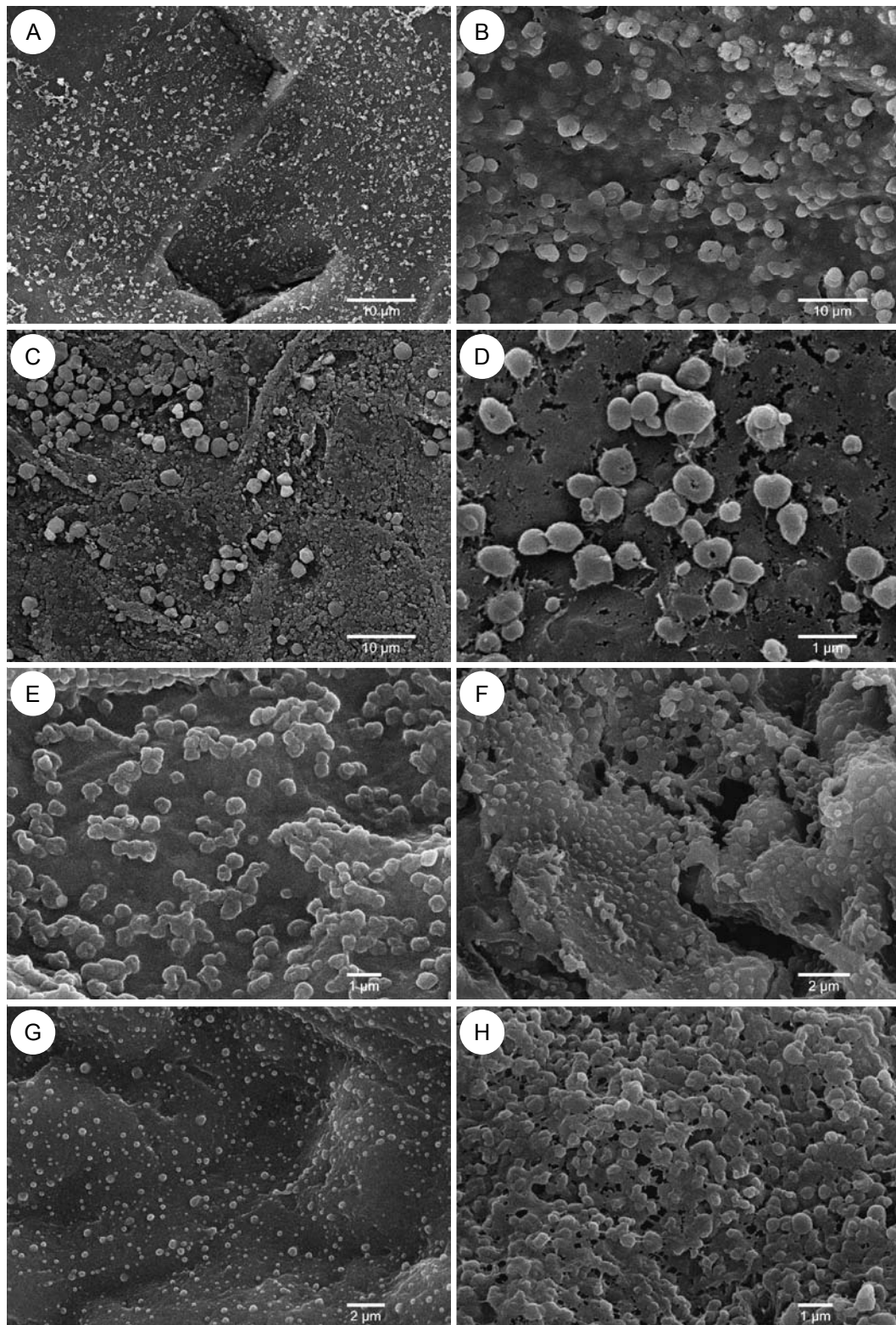
In this study original data for nineteen species were collected: orbicules occur in twelve species, the remaining seven have a smooth inner locule wall without patterned sporopollenin polymerization (see fig. 2A). Orbicules are found lining the locule wall as in other angiosperm families and not intermingled with the pollen grains inside the locule. Morphological characteristics of orbicules are summarized in table 1.

**Density** – If present, orbicules are generally very abundant, completely covering the inner locule wall (e.g. *Annickia letestui*, *Cananga odorata* – fig. 1E, *Oxandra xylopioides*). In *Anaxagorea dolichocarpa* (fig. 1A), *Polyalthia sumatrana* (fig. 1G) and *Mosannonna depressa* orbicules are much less dense, they are scattered on the locule wall without touching each other. Orbicules are abundant in *Klarobelia* (fig. 1B) and *Miliusa*.

**Size and shape** – Orbicules in Annonaceae are always spherical and usually very small with mean diameters ranging from 0.29 µm in *Monodora crispata* (fig. 1F) and *Polyalthia sumatrana* (fig. 1G) to 0.92 µm in *Mosannonna depressa*. Only *Klarobelia cauliflora* produced orbicules that are considerably larger (2.62 µm mean diameter; fig. 1B). These structures were considered as genuine orbicules since they have a distinct surface ornamentation and sometimes a central perforation.

**Ornamentation** – Orbicules in Annonaceae are generally smooth without distinct wall ornamentation (e.g. *Anaxagorea dolichocarpa*, *Crematosperma microcarpum* – fig. 1D, *Oxandra xylopioides*). The large orbicules in *Klarobelia cauliflora* (fig. 1B) are microgranulate: the orbicule wall is covered with tiny granules rendering it a rough texture. Moreover, a single central perforation might appear as in *Cananga odorata* (fig. 1E), *Crematosperma microcarpum* (fig. 1D), and *Miliusa velutina*.

**Association with each other and locule wall** – Whether orbicules appear interconnected or embedded in the tapetal remnants might be affected by the preparation protocol and is therefore prone to artefacts. In *Anaxagorea dolichocarpa* (fig. 1A), *Annickia letestui* and *Oxandra xylopioides* we have observed filaments interconnecting the orbicules. It is unclear on SEM images whether the filaments consist of sporopollenin. Orbicules sometimes appear embedded in the tapetal remnants (as in *Anaxagorea dolichocarpa*, *Annickia letestui*, *Klarobelia cauliflora* (fig. 1B), and *Miliusa velutina*). Only in



**Figure 1** – Orbicules in Annonaceae (SEM): A, *Anaxagorea dolichocarpa*; tiny orbicules, regularly spaced on inner locule wall; B, *Klarobelia cauliflora*; orbicules with microgranulate ornamentation, note the central perforation in several orbicules; C, *Crematosperma microcarpum*; next to the tiny orbicules, larger and more angular structures are present; D, *Crematosperma microcarpum*; detail of orbicules, note the central perforation in several orbicules; E, *Cananga odorata*; orbicules abundant on inner locule wall, aggregates of several orbicules are common, some of them have a central perforation; F, *Monodora crispata*; abundant spherical orbicules with a smooth wall; G, *Maasia sumatrana*; tiny orbicules, regularly spaced on inner locule wall; H, *Polyalthia subcordata*; detail of very dense orbicules, interconnected with thin treads.

**Table 2 – Available data on tapetum characterization and orbicule presence in Annonaceae, based on literature survey and present results.**

+ = present, – = absent, P = parietal tapetum, I = invasive non-syncytial tapetum, A = amoeboid tapetum.

Species	Orbicules	Tapetum
<i>Anaxagorea brevipes</i> Benth.	+ Huysmans et al. 1998	P (I) Gabarayeva 1995
<i>A. dolichocarpa</i> Sprague & Sandwith	+ this study	
<i>Annickia letestui</i> (Le Thomas) Setten & Maas	+ this study	
<i>Annona</i> sp.		A Wunderlich 1954 Parulekar 1970 Bhandari 1971 Furness & Rudall 1998
<i>Annona cherimola</i> Mill.	– this study	
<i>A. glabra</i> L.		P Tsou & Johnson 2003
<i>A. montana</i> Macfad.		P Tsou & Johnson 2003
<i>A. muricata</i> L.	– this study	
<i>Annona muricata</i> Jacq. [as <i>Rollinia mucosa</i> (Jacq.) Baill.]	– Huysmans et al. 2000	P Tsou & Johnson 2003
<i>A. senegalensis</i> Pers. subsp. <i>oulotricha</i> Le Thomas	– this study	
<i>A. squamosa</i> L.		A Wunderlich 1954 P + A Periasamy & Kandasamy 1981
<i>Artabotrys hexapetalus</i> (L.f.) Bhandari	– Huysmans et al., 2000	A Furness & Rudall 1998 P Parulekar 1970 Tsou & Johnson 2003
<i>Asimina triloba</i> (L.) Dunal	– Huysmans et al. 1998	A Tsou & Johnson 2003 P then I Gabarayeva 1992, 1993
<i>Asimina</i> × <i>nashii</i> Kral	– this study	
<i>Cananga</i> (DC.) Hook.f. & Thomson		P + A Furness & Rudall 2001
<i>Cananga odorata</i> (Lam.) Hook.f. & Thomson	+ this study	P Periasamy & Swamy 1959 Parulekar 1970 Tsou & Johnson 2003
<i>Cardiopetalum calophyllum</i> Schldl.	– Huysmans et al. 2000	
<i>Crematosperma microcarpum</i> R.E.Fr.	+ this study	
<i>Cyathocalyx apoensis</i> (Elmer) J.Sinclair	+ Huysmans et al. 2000	
<i>Cymbopetalum baillonii</i> R.E.Fr.	– Huysmans et al. 2000	A Tsou & Fu 2007
<i>C. brasiliense</i> (Vell.) Benth. ex Baill.		A Tsou & Johnson 2003
<i>C. longipes</i> Benth. ex Diels	– Huysmans et al. 2000	
<i>Diclinanona calycina</i> (Diels) R.E.Fr.	– Huysmans et al. 2000	
<i>D. tessmannii</i> Diels	– Huysmans et al. 2000	
<i>Fissistigma latifolium</i> (Dunal) Merr.	– this study	
<i>F. oldhamii</i> (Hemsl.) Merr.		A Tsou & Johnson 2003
<i>Froesiodendron amazonicum</i> R.E.Fr.	– Huysmans et al. 2000	A Tsou & Johnson 2003
<i>Goniothalamus</i> (Blume) Hook.f. & Thomson	– Huysmans et al. 2000	A Parulekar 1970
<i>Klarobelia cauliflora</i> Chatrou	+ this study	
<i>Maasia sumatrana</i> (King) Mols	+ this study	
<i>Miliusa veluti</i> (DC. ex Dunal) Hook.f. & Thomson	+ this study	
<i>M. wightiana</i> Hook.f. & Thomson		P Periasamy & Swamy 1959 Parulekar 1970 Furness & Rudall 2001
<i>Mitrephora williamsii</i> C.B.Rob.	+ Huysmans et al. 2000	

**Table 2 (continued)** – Available data on tapetum characterization and orbicule presence in Annonaceae, based on literature survey and present results.

Species	Orbicules	Tapetum	
<i>Monodora angolensis</i> Welw.	– this study		
<i>M. crispata</i> Engl.	+ this study		
<i>M. minor</i> Engl. & Diels	– this study	A	Tsou & Johnson 2003
<i>Mosannona depressa</i> (Baill.) Chatrou	+ this study		
<i>Oxandra xylopioides</i> Diels	+ this study		
<i>Polyalthia</i> Blume		A	Furness & Rudall 2001
<i>Polyalthia laterifolia</i> King	+ this study		
<i>P. subcordata</i> (Blume) Blume	+ this study		
<i>Porcelia magnifructa</i> (Schery) R.E.Fr.	– Huysmans et al. 2000	A	Tsou & Johnson 2003
<i>P. venezuelensis</i> Pittier	– Huysmans et al. 2000		
<i>Pseudoxandra polyphleba</i> (Diels) R.E.Fr.	– Huysmans et al. 2000		
<i>Pseuduvaria filipes</i> (Lauterb. & K.Schum.) J.Sinclair	+ pers. obs. in Su & Saunders 2003		
<i>P. lignocarpa</i> J.Sinclair	+ pers. obs. in Su & Saunders 2003		
<i>P. macrocarpa</i> (Burck) Y.C.F.Su & R.M.K. Saunders	+ pers. obs. in Su & Saunders 2003		
<i>P. unguiculata</i> (Elmer) Y.C.F.Su & R.M.K.Saunders	+ pers. obs. in Su & Saunders 2003		
<i>Trigynaea duckei</i> (R.E.Fr.) R.E.Fr.	– Huysmans et al. 2000		
<i>Uvaria rufa</i> Blume	– Huysmans et al. 2000		
<i>Xylopia africana</i> Oliv.	– Huysmans et al. 2000		
<i>X. aromatica</i> Mart.	– Huysmans et al. 2000		
<i>X. calophylla</i> R.E.Fr.	– Huysmans et al. 2000		
<i>X. collina</i> Diels	– Huysmans et al. 2000		
<i>X. ferruginea</i> (Hook.f. & Thomson) Hook.f. & Thomson	– Huysmans et al. 2000		
<i>X. mwasumbii</i> D.M. Johnson	– Huysmans et al. 2000		

*Cananga odorata* (fig. 1E) orbicules were sometimes found to aggregate, forming clusters of several fused orbicules.

Prior to the new data presented here, the presence/absence of orbicules was known in 24 species of Annonaceae (Huysmans et al. 2000). Moreover, we noticed the presence of orbicules in four *Pseuduvaria* species based on Su & Saunders (2003). This brings the total number of Annonaceae species with orbicules to nineteen, and the number of species lacking them to 28 (table 2).

#### Unidentified (tapetal?) secretions

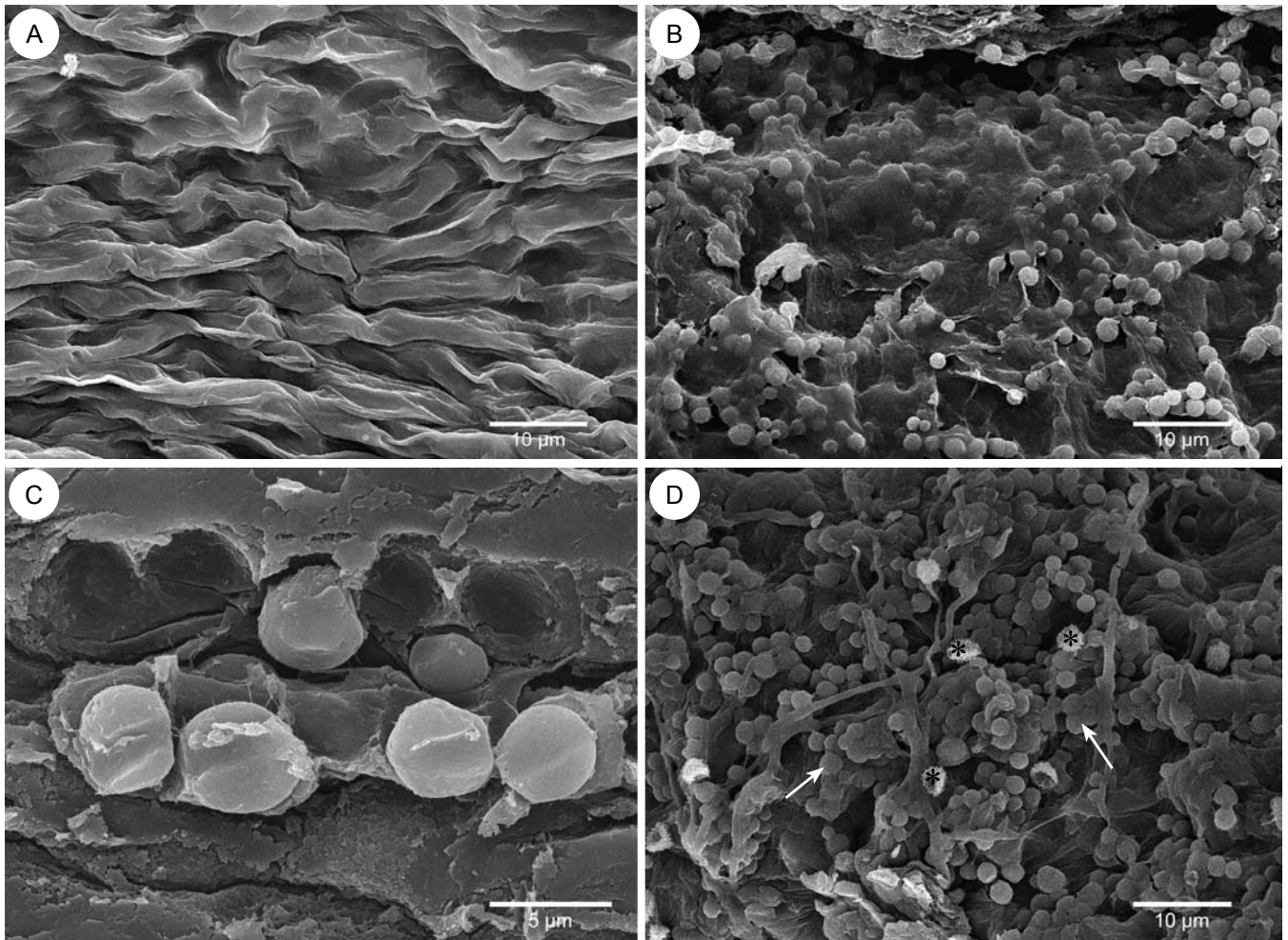
While observing presence/absence of orbicules in SEM, we regularly came across globular structures that are larger than the average Annonaceae orbicules (up to 4 µm in diameter, fig. 2C). Their density was low and restricted to certain areas of the locule wall (compare fig. 2A & B). Based on those two deviating characteristics, we concluded that they do not represent genuine orbicules (fig. 2D). In *Monodora minor* orbicules are absent but globules with a rough surface appear here and there on the locule wall (fig. 3D). A cross section of the anther wall shows similar structures inside the endothecium cells (fig. 3E). These globular structures were observed in

*Annona cherimola*, *A. muricata*, *Asimina* × *nashii*, *Crematosperma microcarpum*, *Fissistigma latifolium*, *Monodora crispata*, *M. minor* and *Mosannona depressa*. The globules are sometimes reminiscent of the raspberry-like silica deposits found in several Commelinales families (Prychid et al. 2003). Most likely they are starch grains that together with druses are released from the middle layers and the septa (see also Tsou & Johnson 2003).

#### Correlation between orbicule presence and tapetum type

An extensive literature search resulted in tapetum data for seventeen species from fourteen genera, of which nine species are also documented for orbicule presence/absence (table 2). In Annonaceae all three main tapetum types were recorded which is a rather exceptional situation for Magnoliales and basal angiosperms in general. Detailed analysis of table 2 reveals few cases of conflicting tapetum type designations between different papers (e.g. *Artabotrys hexapetalus*, *Asimina triloba*). Several studies reported that parietal tapetal cells tend to invade the locule at a certain phase during development (e.g. in *Anaxagorea brevipes*, *Asimina triloba*). Our compilation of the scarce tapetum data in Annonaceae clearly





**Figure 2** – Taxa without genuine orbicules but with globular structures (SEM): A, *Annona cherimola*; inner locule wall is smooth, orbicules are lacking, ridges of endothecium beneath are visible; B, *Annona cherimola*; on some parts of locule wall spherical globules are present; C, *Annona muricata*; relatively large globular structures on inner locule wall. Mainly because of large size and low density they are not considered to be orbicules; D, *Fissistigma latifolium*; view on locule wall with fungi hyphen and some fungi spores (asterisks) and many globular structures (arrows) not considered to be orbicules.

indicates the plasticity and highly dynamic nature of this tissue. Of the nine documented species, four are lacking orbicules and have an amoeboid tapetum. Three species have a parietal tapetum of which two species produce orbicules. For the two other species tapetum data are conflicting but they are both lacking orbicules (table 2). Thus, the hypothesis that orbicules are restricted to non-amoeboid tapetum types holds true for Annonaceae.

### Character optimisation

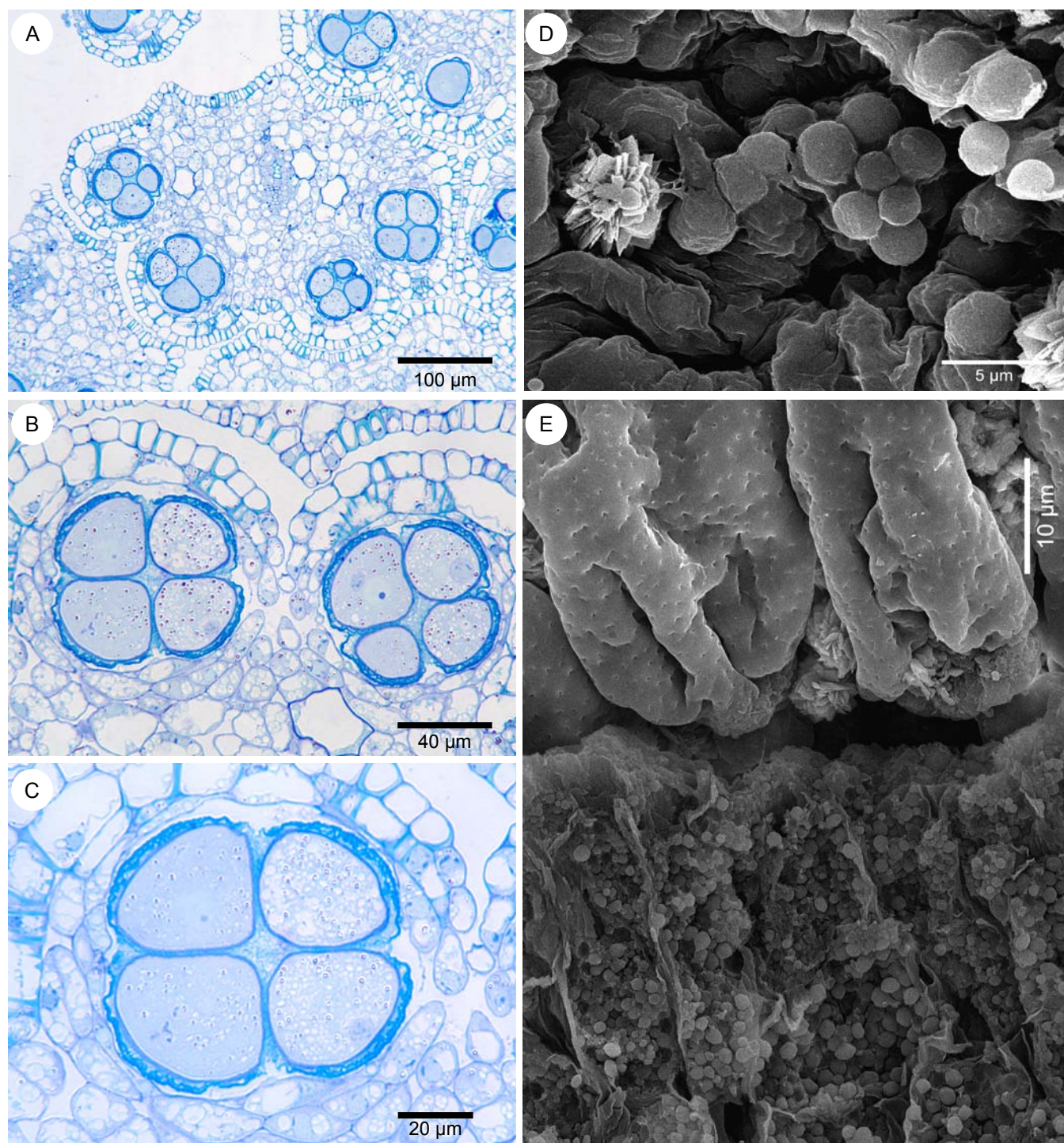
Optimisation of presence/absence of orbicules was unambiguous, with the exception of two cases (fig. 4). All nodes of the *Malmea/Pseudoxandra/Crematosperma* clade are ambiguously resolved, as well as of a clade of eleven African genera in the long branch clade. The ambiguity extends down to the stem nodes of these two clades, which is caused by the lack of orbicule observations of genera closely related to *Pseudoxandra* and *Monodora*.

## DISCUSSION

### Phylogenetic signal of orbicule distribution

The most recent hypothesis of evolutionary relationships in Annonaceae (Chatrou et al. subm.) shows four major clades (fig. 4) with the majority of the species belonging to the ‘short branch clade’ (c. 800 species) or the ‘long branch clade’ (c. 1500 species). The latter clade is characterized by an elevated substitution rate in the chloroplast genome resulting in average branch lengths (from common ancestor to all terminals) that double those in the short branch clade. The increased substitution rates in the cpDNA matches the evolution of some life history features that are restricted to the ‘long branch clade’, e.g. a climbing habit and (pseudo-)syncarpous fruits. In other words, the plesiomorphic character states (tree-like habit, apocarpous fruits) are present in *Anaxagorea*, the ambavioids and the short branch clade.

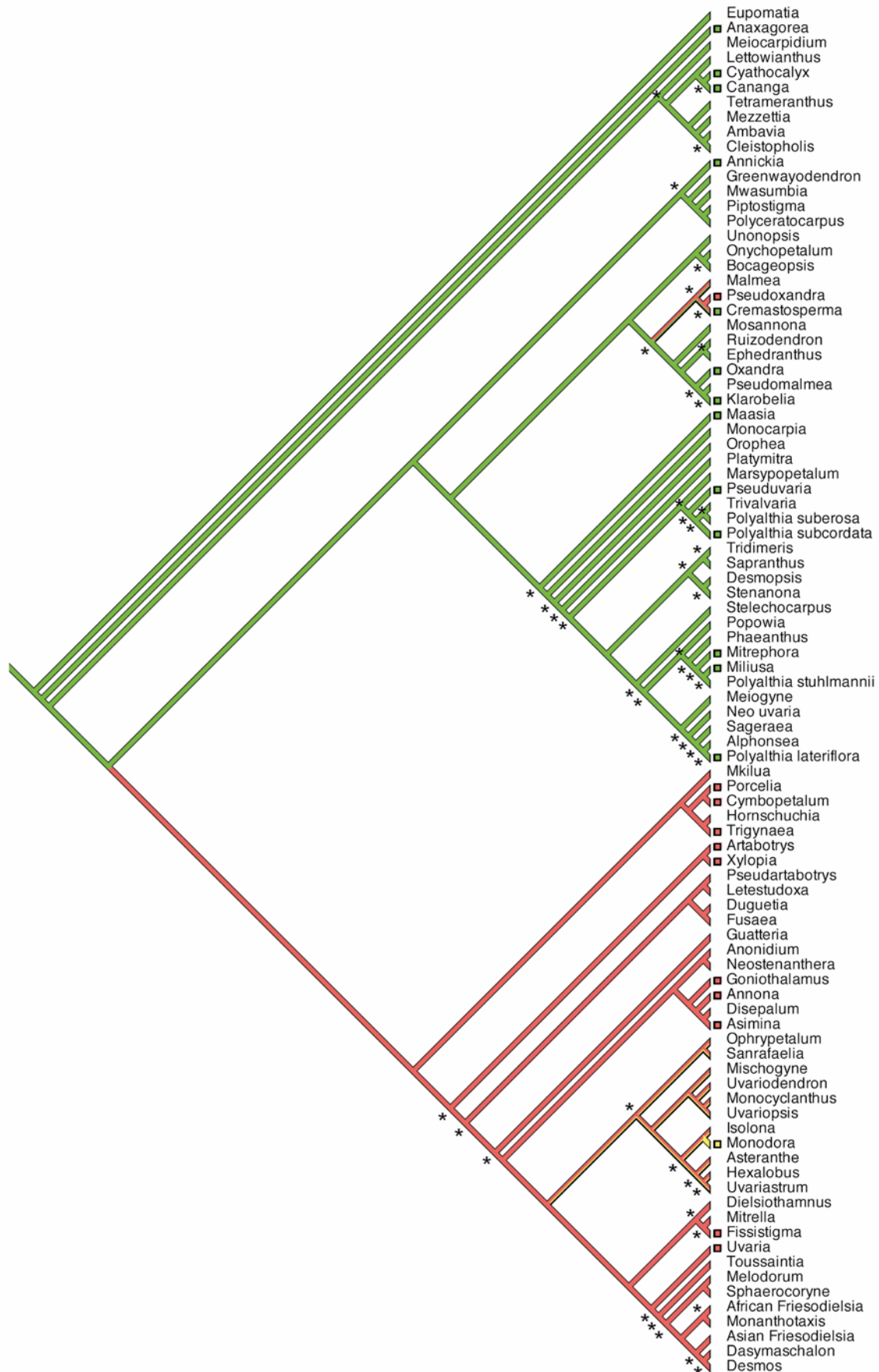
Orbicule distribution on the phylogenetic tree shows a similar pattern: orbicules are present in *Anaxagorea*, the am-



**Figure 3** – *Monodora* anther morphology: A–C, *Monodora angolensis* (LM); cross sections of densely packed anthers with details of permanent tetrads; tetrads occur in a single row in each locule; orbicules are absent; D–E, *Monodora minor* (SEM); D, roughly textured globules on inner locule wall with at left hand side a druse; E, cross section through mature anther showing dehydrated pollen on top of anther wall tissue. Note many similar globules inside the cells.

**Figure 4** – Cladogram, based on Chatrou et al. (subm.), showing relationships amongst 88 genera of Annonaceae with optimization of presence (green) and absence (red) of orbicules. Yellow indicates the co-occurrence of both states within a single genus. Coloured squares above the tips designate actual observations of presence/absence of orbicules Asterisks indicate clades that receive less than 85% bootstrap support in Chatrou et al. (subm.). *Eupomatia* (Eupomatiaceae) is the sister group of Annonaceae.





bavioids and the short branch clade, and are absent in the long branch clade, illustrating the plesiomorphic nature of this feature within Annonaceae (fig. 4). The 'long branch clade' is entirely lacking orbicules, which is most likely pointing to a shift in tapetum type from a parietal to a plasmodial type in the common ancestor of this clade. Under the constraint that we only have data from 27 out of 111 currently recognized genera, the distribution of orbicule presence/absence is very consistent with phylogenetic patterns in Annonaceae. There are two notable exceptions that prevent a perfect match onto the phylogenetic tree in fig. 4. That is, if we ignore the ambiguously resolved nodes in the tree, we can assume a reversal to the plesiomorphic condition (in *Monodora*), and a parallel loss of orbicules (in *Monodora*). Assuming these two changes in the two genera mentioned is the most conservative hypothesis we can put forward. Further observations on closely related genera would be needed to test this. In *Pseudoxandra* (data of two species available) orbicules were absent although the genus is part of the 'short branch clade'. Moreover, the present study identified *Monodora crispata* to produce orbicules, forming the only exception in the 'long branch clade'. However, *Monodora minor* and *M. angolensis* are lacking orbicules. The observed variability within *Monodora* cannot be attributed to taxonomic uncertainties. An updated taxonomy as well as phylogenetic relationships of *Monodora* have recently been published (Couvreur 2009), making use of the very same collections that we sampled in this study. Apart from the discrepancy with other long branch clade taxa, this observation, therefore, denotes the only disagreement in Annonaceae with the general observation of constancy of presence/absence of orbicules on generic level in flowering plants (Huysmans et al. subm.).

Three genera mentioned in table 2, viz. *Cardiopetalum*, *Froesiodendron*, and *Diclinanona* are not represented in fig. 4. Morphological-cladistic analyses have put the former two genera in the tribe Bocageae (Johnson & Murray 1995), here represented by *Mkilua*, *Cymbopetalum*, and *Porcelia*. Relationships of *Cardiopetalum* and *Froesiodendron* are confirmed by the absence of orbicules. The phylogenetic position of *Diclinanona* has been a matter of debate. Richardson et al. (2004) inferred this genus to be included in the long branch clade, sister to a large clade of African genera of trees and Paleotropical genera of lianas. Erkens et al. (2009) contested this position, based on laboratory related errors, and inferred a short branch clade relationship for *Diclinanona*. Awaiting confirmation by newly generated DNA sequences, orbicule absence in this genus predicts an affiliation with the long branch clade.

Eupomatiaceae is the sister group of Annonaceae and the single species (*Eupomatia laurina* R.Br.) studied indeed has orbicules (Huysmans et al. 1998). Our data convincingly show that orbicule presence is a plesiomorphic feature in Annonaceae, and add to the derived nature of the long branch clade. There is a single loss of orbicules along the branch subtending this clade, affecting well over half of the species in the family, and a second loss in the Neotropical genus *Pseudoxandra*. Remarkably, *Pseudoxandra* is also the sole genus in the 'short branch clade' with pollen dispersal in permanent tetrads (Doyle & Le Thomas 1994; see also Harder & Johnson 2008); all other representatives shed their pollen

in monads (considered the basic type). Thus derived lineages are missing orbicules while they are generally present in the basal clades, a pattern that is also recognized in other angiosperm groups such as monocots, asterids and Rubiaceae (Verstraete et al. subm.), and in angiosperms overall (Huysmans et al. subm.).

### Correlation of orbicule production with other staminal features in Annonaceae

The co-occurrence of both positive and negative observations for orbicule presence in a single family is rather uncommon in angiosperms as a whole. A recent review of all available data in flowering plants recovered only nineteen out of 142 documented families with both presence and absence of orbicules (Huysmans et al. subm.). The evolutionary constraints and selective pressures that affect the mere presence/absence of patterned sporopollenin polymerisation in the form of orbicules remain unknown to date. Our results for orbicule distribution and tapetum differentiation clearly indicate that Annonaceae may represent an ideal case to further our knowledge. Moreover, this family is within the magnoliids the most diversified lineage palynologically. Most of the types of apertures, pollen wall architecture and pollen dispersal units within magnoliids as a whole, occur within Annonaceae (Sampson 2000).

**Tapetum type** – In Annonaceae an extensive tapetum diversity is reported (Furness & Rudall 2001), including parietal, amoeboid and non-plasmodial invasive types, which often triggers orbicule variation (see table 2). This wide tapetum diversity in a single family is exceptional, even in basal angiosperms. Basal angiosperms predominantly have parietal tapeta, plasmodial and invasive types have a more restricted distribution among these taxa. Only in Atherospermataceae, Hernandiaceae and Lauraceae (Laurales) and possibly Winteraceae (Cannellales) tapetum types other than parietal were documented (Furness & Rudall 2001). Furness & Rudall (2001: 390) speculated carefully that "this apparent plasticity may represent a similar range of evolutionary experimentation in closely related groups for a relatively brief but critical time in angiosperm evolution, perhaps in connection with early evolution of highly specific pollination syndromes".

**Pollen dispersal unit** – Orbicules have never been observed in taxa with massulae or compact pollinia (Pacini 1997). They are, however, present in *Acacia* species that develop polyads (Kenrick & Knox 1979; G. Prenner, RBG Kew, U.K., pers. comm.). Correlation of orbicule presence with pollen dispersal in permanent tetrads has never been analysed. In Annonaceae pollen may be dispersed as monads, in permanent tetrads or in polyads (Sampson 2000). Intriguing in the present results is the absence of orbicules in the Neotropical genus *Pseudoxandra*, the sole genus in the 'short branch clade' with pollen dispersal in permanent tetrads. All other genera investigated in the 'short branch clade' do produce orbicules and shed their pollen as monads. *Cananga odorata* (ambavioids) sheds its pollen in tetrads and produces abundant orbicules (this study).

**Septate anthers** – In Annonaceae both nonseptate (basic type) and septate anthers occur (see Tsou & Johnson 2003 for a review). Two septa types were described depending on the

number of cell layers involved (with or without central parenchymatous cell layer) and they both have a distinct tapetal function. Both types are considered associated with the production of compound pollen (Tsou & Johnson 2003) which leads us back to the previous paragraph. To date the available data are too scarce to elaborate on a possible correlation between tapetal lipid metabolism (initiation of pro-orbicules) and the spatial organisation pattern of both tapetal cells (septa or not) and microspores (aggregated pollen or not).

## CONCLUSIONS

Annonaceae is one of the few families in angiosperms with both presence and absence of orbicules reflecting the high diversity of tapetum types within this group. The distribution pattern of orbicules in Annonaceae reflects the general pattern observed in flowering plants: orbicules are a plesiomorphic feature, common in the early diverging lineages with a trend towards orbicule-absence in the more derived clades. Other secretions similar in form to orbicules but usually larger and less dense are encountered regularly in Annonaceae anthers. They probably represent starch granules released from middle layers and stamen septa. The hypothesis that orbicules are restricted to non-amoebooid tapetum types holds true for Annonaceae. The presence of orbicules can be considered as a powerful proxy for non-amoebooid tapetum characterization in the family. Our results demonstrate the potential of Annonaceae as a case study in the early diverging lineages of angiosperms for tapetum and orbicule research.

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## REFERENCES

- Bhandari N.N. (1971) Embryology of the Magnoliales and comments on their relationships. *Journal of the Arnold Arboretum* 52: 1–39, 285–304.
- Blackmore S., Wortley A.H., Skvarla J.J., Rowley J.R. (2007) Pollen wall development in flowering plants. *New Phytologist* 174: 483–498.
- Chatrou L.W., Rainer H., Maas P.J.M. (2004) Annonaceae. In: Smith N., Mori S.A., Henderson A., Stevenson D.W., Heald S.V. (eds) *Flowering plants of the Neotropics*: 18–20. Princeton, Princeton University Press.
- Chatrou L.W., Pirie M.D., Couvreur T.L.P., Erkens R.H.J., Mols J.B., Bygrave P., Maas J.W., Wang J., Zhou L., Saunders R.M.K., Chase M.W. (submitted) Phylogeny and classification of the pantropical flowering plant family Annonaceae: evidence from multiple chloroplast loci. *American Journal of Botany*.
- Christensen J.E., Horner Jr. H.T., Lersten N.R. (1972) Pollen wall and tapetal orbicular wall development in *Sorghum bicolor* (Gramineae). *American Journal of Botany* 59: 43–58.
- Couvreur T.L.P., Richardson J.E., Erkens R.H.J., Sosef M.S.M., Chatrou, L.W. (2008) Evolution of syncarpy and other morphological characters in African Annonaceae: a posterior mapping approach. *Molecular Phylogenetics and Evolution* 47: 302–318.
- Couvreur T.L.P. (2009) Monograph of the syncarpous African genera *Isolona* and *Monodora* (Annonaceae). *Systematic Botany Monographs* 87: 1–150.
- Dessein S., Ochoterena H., De Block P., Lens F., Robbrecht E., Schols P., Smets E., Vincier S. (2005) Palynological characters and their phylogenetic signal in Rubiaceae. *The Botanical Review* 71: 354–414.
- Doyle J.A., Le Thomas A. (1994) Cladistic analysis and pollen evolution in Annonaceae. *Acta Botanica Gallica* 141: 149–170.
- El-Ghazaly G., Jensen W.A. (1986) Studies of the development of wheat (*Triticum aestivum*) pollen. I. Formation of the pollen wall and Ubisch bodies. *Grana* 25: 1–29.
- El-Ghazaly G., Huysmans S. (2001) Re-evaluation of a neglected layer in pollen wall development with comments on its evolution. *Grana* 40: 3–16.
- Erkens R.H.J., Maas J.W., Couvreur T.L.P. (2009) From Africa via Europe to South America: migrational route of a species-rich genus of Neotropical lowland rain forest trees (*Guatteria*, Annonaceae). *Journal of Biogeography* 36: 2338–2352.
- Fitch W.M. (1971) Towards defining the course of evolution: Minimum change for a specific tree topology. *Systematic Zoology* 20:406–416.
- Furness C.A., Rudall P.J. (1998) The tapetum and systematics in monocotyledons. *The Botanical Review* 64: 201–239.
- Furness C.A., Rudall P.J. (2001) The tapetum in basal angiosperms: early diversity. *International Journal of Plant Sciences* 162: 375–392.
- Gabarayeva N.I. (1992) Sporoderm development in *Asimina triloba* (Annonaceae). I. The developmental events before callose dissolution. *Grana* 31: 213–222.
- Gabarayeva N.I. (1993) Sporoderm development in *Asimina triloba* (Annonaceae). II. The developmental events after callose dissolution. *Grana* 32: 210–220.
- Gabarayeva N.I. (1995) Pollen wall and tapetum development in *Anaxagorea brevipes* (Annonaceae): sporoderm substructure, cytoskeleton, sporopollenin precursor particles, and the endexine problem. *Review of Palaeobotany and Palynology* 85: 123–152.
- Harder L.D., Johnson S.D. (2008) Function and evolution of aggregated pollen in angiosperms. *International Journal of Plant Sciences* 169: 59–78.
- Huysmans S., El-Ghazaly G., Nilsson S., Smets E. (1997) Systematic value of tapetal orbicules: a preliminary survey of the Cinchonoideae (Rubiaceae). *Canadian Journal of Botany* 75: 815–826.
- Huysmans S., El-Ghazaly G., Smets E. (1998) Orbicules in angiosperms. Morphology, function, distribution, and relation with tapetum types. *The Botanical Review* 64: 240–272.
- Huysmans S., El-Ghazaly G., Smets E. (2000) Orbicules: still a well hidden secret of the anther. In: Nordenstam B., El-Ghazaly G., Kassas M. (eds) *Plant systematics for the 21<sup>st</sup> century*: 201–212. London, Portland Press.

- Huysmans S., Verstraete B., Moon H.-K., Smets E.F. (submitted) Orbicules in flowering plants: a phylogenetic perspective on form and function. *American Journal of Botany*.
- Johnson D.M., Murray N.A. (1995) Synopsis of the tribe Bocageae (Annonaceae), with revisions of *Cardiopetalum*, *Froesiodendron*, *Trigynaea*, *Bocagea*, and *Hornschuchia*. *Brittonia* 47: 248–319.
- Kenrick J., Knox R.B. (1979) Pollen development and cytochemistry in some Australian species of *Acacia*. *Australian Journal of Botany* 27: 413–427.
- Kosmuth L. von. (1927) Studien über das Antherentapetum. *Österreichische Botanische Zeitschrift* 76: 235–241.
- Maddison W.P., Maddison D.R. (2009) Mesquite: a modular system for evolutionary analysis, v. 2.72. <http://mesquiteproject.org>
- Mols J.B., Gravendeel B., Chatrou L.W., Pirie M.D., Bygrave P., Chase M.W., Kessler P.J.A. (2004) Identifying clades in Asian Annonaceae: monophyletic genera in the polyphyletic Miliuseae. *American Journal of Botany* 91: 590–600.
- Mols J.B., Kessler P.J.A., Rogstad S.H., Saunders R.M.K. (2008) Reassignment of six *Polyalthia* species to the new genus *Maasia* (Annonaceae): Molecular and morphological congruence. *Systematic Botany* 33: 490–494.
- Pacini E., Franchi G.G., Hesse M. (1985) The tapetum: its form, function and possible phylogeny in Embryophyta. *Plant Systematics and Evolution* 149: 155–185.
- Pacini E. (1997) Tapetum character states: analytical keys for tapetum types and activities. *Canadian Journal of Botany* 75: 1448–1459.
- Pacini E. (2010) Relationships between tapetum, loculus, and pollen during development. *International Journal of Plant Sciences* 171: 1–11.
- Parulekar N.K. (1970) Annonaceae. *Bulletin of Indian National Academy of Sciences* 41: 38–41.
- Periasamy K., Swamy B.G.L. (1959) Studies in the Annonaceae. I. Microsporogenesis in *Cananga odorata* and *Miliusa wrightiana*. *Phytomorphology* 9: 251–263.
- Periasamy K., Kandasamy M.K. (1981) Development of the anther of *Annona squamosa* L. *Annals of Botany* 48: 885–893.
- Pirie M.D., Chatrou L.W., Mols J.B., Erkens R.H.J., Oosterhof J. (2006) ‘Andean-centred’ genera in the short-branch clade of Annonaceae: testing biogeographic hypotheses using phylogeny reconstruction and molecular dating. *Journal of Biogeography* 33: 31–46.
- Prychid C.J., Furness C.A., Rudall P.J. (2003) Systematic significance of cell inclusions in Haemodoraceae and allied families: silica bodies and tapetal raphides. *Annals of Botany* 92: 571–580.
- Richardson J.E., Chatrou L.W., Mols J.B., Erkens R.H.J., Pirie M.D. (2004) Historical biogeography of two cosmopolitan families of flowering plants: Annonaceae and Rhamnaceae. *Philosophical Transactions of the Royal Society B: Biological Sciences* 359: 1495–1508.
- Rosanoff S. (1865) Zur Kenntnis des Baues und der Entwicklungsgeschichte des Pollens der Mimoseae. *Jahrbuch für wissenschaftliche Botanik* 4: 441–450.
- Rowley J.R., Gabarayeva N.I., Walles B. (1992) Cyclic invasion of tapetal cells into loculi during microspore development in *Nymphaea colorata* (Nymphaeaceae). *American Journal of Botany* 79: 801–808.
- Sampson F.B. (2000) Pollen diversity in some modern magnoliids. *International Journal of Plant Sciences* 161: S193–S210.
- Sauquet H., Doyle J.A., Scharaschkin T., Borsch T., Hilu K.W., Chatrou L.W., Le Thomas A. (2003) Phylogenetic analysis of Magnoliales and Myristicaceae based on multiple data sets: implications for character evolution. *Botanical Journal of the Linnean Society* 142: 125–186.
- Schols P., Dessein S., D’Hondt C., Huysmans S., Smets E. (2002) Carnoy: a new digital measurement tool for palynology. *Grana* 41: 124–126.
- Scott R.J. (1994) Pollen exine – the sporopollenin enigma and the physics of pattern. In: Scott R.J., Stead M.A. (eds) *Molecular and cellular aspects of plant reproduction*: 49–81. Cambridge, Cambridge University Press.
- Su Y.C.F., Saunders R.M.K. (2003) Pollen structure, tetrad cohesion and pollen-connecting threads in *Pseuduvaria* (Annonaceae). *Botanical Journal of the Linnean Society* 143: 69–78.
- Tsou C.-H., Johnson D.M. (2003) Comparative development of aseptate and septate anthers of Annonaceae. *American Journal of Botany* 90: 832–848.
- Tsou C.-H., Fu Y.-L. (2007) Octad pollen formation in *Cymbopetalum* (Annonaceae): the binding mechanism. *Plant Systematics and Evolution* 263: 13–23.
- Ubisch G. von. (1927) Zur Entwicklungsgeschichte der Antheren. *Planta* 3: 490–495.
- Verstraete B., Groeninckx I., Smets E.F., Huysmans S. (submitted) Evolution of orbicules in Rubiaceae. *Annals of Botany*.
- Vinckier S., Huysmans S., Smets E. (2000) Morphology and ultrastructure of orbicules in the subfamily Ixoroideae (Rubiaceae). *Review of Palaeobotany and Palynology* 108: 151–174.
- Wunderlich R. (1954) Über das Antherentapetum mit besonderer Berücksichtigung seiner Kernzahl. *Österreichische Botanische Zeitschrift* 101: 1–63.
- Zhou L., Su Y.C.F., Saunders R.M.K. (2009) Molecular phylogenetic support for a broader delimitation of *Uvaria* (Annonaceae), inclusive of *Anomianthus*, *Cyathostemma*, *Ellipeia*, *Ellipeiopsis* and *Rauwenhoffia*. *Systematics and Biodiversity* 7: 249–258.

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**Appendix – Collections sampled for the present study.**

Material in ethanol is indicated by \*, living material by °, all others are herbarium specimens.

- Anaxagorea dolichocarpa* Sprague & Sandwith, Guyana, *P.J.M. Maas* 2432 (U)\*  
*Annickia letestui* (Le Thomas) Setten & Maas, Cameroon, *L.W. Chatrou* 568 (WAG)\*  
*Annona cherimola* Mill., Ethiopia, *P.C.M. Jansen* 5206 (WAG)  
*A. muricata* L., Nigeria, *C.L.M. van Eijnatten* 1442 (WAG)  
*A. senegalensis* Pers. subsp. *oulotricha* Le Thomas, Cameroon, *S.N. Ekema* 138 (WAG)  
*Asimina* × *nashii* Kral, USA, *J.R. Abbott* & *B. Carlsward* 14289 (U)  
*Cananga odorata* (Lam.) Hook.f. & Thomson, Costa Rica, *G. Herrera* 1754 (U)  
*Crematosperma microcarpum* R.E.Fr., Peru, *P.J.M. Maas* 6281 (U)\*  
*Fissistigma latifolium* (Dunal) Merr., Indonesia, *R.C. Bakhuizen-van den Brink Jr.* s.n. (WAG)  
*Isolona cooperi* Hutch. & Dalziel ex G.P.Cooper & Record, cultivated in National Botanic Garden of Belgium, 19820471°  
*Klarobelia cauliflora* Chatrou, Peru, *L.W. Chatrou* 161 (U)\*  
*Maasia. sumatrana* (King) Mols, P.J.A. Kefler & S. H. Rogstad, Indonesia, *Bosch proefstation* T266 (WAG)  
*Miliusa velutina* (DC. ex Dunal) Hook.f. & Thomson, Thailand, *L.W. Chatrou* & *U. Thongpairaj* 625 (WAG)  
*Monanthotaxis whytei* (Stapf) Verdc., cultivated in National Botanic Garden of Belgium, 19820471°  
*Monodora angolensis* Welw., cultivated in National Botanic Garden of Belgium, 19514759°  
*M. crispata* Engl., Cameroon, *J.J. Bos* 6224 (WAG)  
*M. minor* Engl. & Diels, Tanzania, *T.L.P. Couvreur* 36 (WAG)  
*Mosannonna depressa* (Baill.) Chatrou, Guatemala, *H. Zomer* 201 (U)\*  
*Oxandra xylopioides* Diels, Peru, *M.D. Pirie* 30 (U)\*  
*Polyalthia laterifolia* King, Indonesia, *S.H. Koorders* 20452B (WAG)  
*P. subcordata* (Blume) Blume, Indonesia, *van Balgooy* & *van Setten* 5667 (U)
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